TITLE OF THE INVENTION

PLATELET-DERIVED GROWTH FACTOR D, DNA CODING THEREFOR, AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. Application No. 09/691,200, filed October 19, 2000, which is a continuation-in-part of U.S. Application No. 09/438,046, filed November 10, 1999 and claims the benefit of U.S. Provisional Application No. 60/107,852, filed November 10, 1998; U.S. Provisional Application No. 60/113,997, filed December 28, 1998; U.S. Provisional Application No. 60/150,604, filed August 26, 1999; U.S. Provisional Application No. 60/157,108, filed October 4, 1999; and U.S. Provisional Application No. 60/157,756, filed October 5, 1999.

FIELD OF THE INVENTION

[0002] This invention relates to growth factors for cells expressing receptors to a novel growth factor that include endothelial cells, connective tissue cells (such as fibroblasts) myofibroblasts and glial cells, and in particular to a novel platelet-derived growth factor/ vascular endothelial growth factor-like growth factor, polynucleotide sequences encoding the factor, and to pharmaceutical and diagnostic compositions and methods utilizing or derived from the factor.

BACKGROUND OF THE INVENTION

[0003] In the developing embryo, the primary vascular network is established by *in situ* differentiation of mesodermal cells in a process called vasculogenesis. It is believed that all subsequent processes involving the generation of new vessels in the embryo and neovascularization in adults, are governed by the sprouting or splitting of new capillaries from the pre-existing vasculature in a process called angiogenesis (Pepper *et al.*, Enzyme & Protein, 1996 49 138-162; Breier *et al.*, Dev. Dyn. 1995 204 228-239; Risau, Nature, 1997 386 671-674). Angiogenesis is not only involved in embryonic development and normal tissue growth, repair, and regeneration, but is also involved in the female reproductive cycle, establishment and maintenance of pregnancy, and in repair of wounds and fractures. In addition to angiogenesis which takes place in the normal individual, angiogenic events are involved in a number of pathological processes, notably tumor growth and metastasis, and other conditions in which blood vessel proliferation, especially of the microvascular system, is increased, such as diabetic retinopathy, psoriasis and arthropathies. Inhibition of angiogenesis is useful in preventing or alleviating these pathological processes.

[0004] On the other hand, promotion of angiogenesis is desirable in situations where vascularization is to be established or extended, for example after tissue or organ transplantation, or to stimulate establishment of collateral circulation in tissue infarction or arterial stenosis, such as in coronary heart disease and thromboangitis obliterans.

[0005] The angiogenic process is highly complex and involves the maintenance of the endothelial cells in the cell cycle, degradation of the extracellular matrix, migration and invasion of the surrounding tissue and finally, tube formation. The molecular mechanisms underlying the complex angiogenic processes are far from being understood.

[0006] Because of the crucial role of angiogenesis in so many physiological and pathological processes, factors involved in the control of angiogenesis have been intensively investigated. A number of growth factors have been shown to be involved in the regulation of angiogenesis; these include fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), transforming growth factor alpha (TGF), and hepatocyte growth factor (HGF). See for example Folkman *et al.*, J. Biol. Chem., 1992 267 10931-10934 for a review.

[0007] It has been suggested that a particular family of endothelial cell-specific growth factors, the vascular endothelial growth factors (VEGFs), and their corresponding receptors is primarily responsible for stimulation of endothelial cell growth and differentiation, and for certain functions of the differentiated cells. These factors are members of the PDGF family, and appear to act primarily via endothelial receptor tyrosine kinases (RTKs).

[0008] Eight different proteins have been identified in the PDGF family, namely two PDGFs (A and B), VEGF and five members that are closely related to VEGF. The five members closely related to VEGF are: VEGF-B, described in

International Patent Application PCT/US96/02957 (WO 96/26736) which corresponds to U.S. Patent 5,928,939 and in U.S. Patents 5,840,693 and 5,607,918 by Ludwig Institute for Cancer Research and The University of Helsinki; VEGF-C or VEGF-2, described in Joukov *et al.*, EMBO J., 1996 15 290-298 and Lee *et al.*, Proc. Natl. Acad. Sci. USA, 1996 93 1988-1992, and U.S. Patents 5,932,540, 5,935,820 and 6,040,157; VEGF-D, described in International Patent Application No. PCT/US97/14696 (WO 98/07832), and Achen *et al.*, Proc. Natl. Acad. Sci. USA, 1998 95 548-553; the placenta growth factor (PlGF), described in Maglione *et al.*, Proc. Natl. Acad. Sci. USA, 1991 88 9267-9271; and VEGF3, described in International Patent Application Nos. PCT/US95/07283 (WO 96/39421) and PCT/US99/18054 (WO 00/09148) by Human Genome Sciences, Inc. Each VEGF family member has between 30% and 45% amino acid sequence identity with VEGF. The VEGF family members share a VEGF homology domain which contains the six cysteine residues which form the cysteine knot motif. Functional characteristics of the VEGF family include varying degrees of mitogenicity for endothelial cells, induction of vascular permeability and angiogenic and lymphangiogenic properties.

[0009] Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein that has been isolated from several sources. VEGF shows highly specific mitogenic activity for endothelial cells. VEGF has important regulatory functions in the formation of new blood vessels during embryonic vasculogenesis and in angiogenesis during adult life (Carmeliet *et al.*, Nature, 1996 380 435-439;

Ferrara *et al.*, Nature, 1996 380 439-442; reviewed in Ferrara and Davis-Smyth, Endocrine Rev., 1997 18 4-25). The significance of the role played by VEGF has been demonstrated in studies showing that inactivation of a single VEGF allele results in embryonic lethality due to failed development of the vasculature (Carmeliet *et al.*, Nature, 1996 380 435-439; Ferrara *et al.*, Nature, 1996 380 439-442). In addition VEGF has strong chemoattractant activity towards monocytes, can induce the plasminogen activator and the plasminogen activator inhibitor in endothelial cells, and can also induce microvascular permeability. Because of the latter activity, it is sometimes referred to as vascular permeability factor (VPF). The isolation and properties of VEGF have been reviewed; see Ferrara *et al.*, J. Cellular Biochem., 1991 47 211-218 and Connolly, J. Cellular Biochem., 1991 47 219-223. Alterative mRNA splicing of a single VEGF gene gives rise to five isoforms of VEGF.

[0010] VEGF-B has similar angiogenic and other properties to those of VEGF, but is distributed and expressed in tissues differently from VEGF. In particular, VEGF-B is very strongly expressed in heart, and only weakly in lung, whereas the reverse is the case for VEGF. This suggests that VEGF and VEGF-B, despite the fact that they are co-expressed in many tissues, may have functional differences.

[0011] VEGF-B was isolated using a yeast co-hybrid interaction trap screening technique by screening for cellular proteins which might interact with

cellular resinoid acid-binding protein type I (CRABP-I). Its isolation and characteristics are described in detail in PCT/US96/02957 and in Olofsson *et al.*, Proc. Natl. Acad. Sci. USA, 1996 93 2576-2581.

[0012] VEGF-C was isolated from conditioned media of the PC-3 prostate adenocarcinoma cell line (CRL1435) by screening for ability of the medium to produce tyrosine phosphorylation of the endothelial cell-specific receptor tyrosine kinase VEGFR-3 (Flt4), using cells transfected to express VEGFR-3. VEGF-C was purified using affinity chromatography with recombinant VEGFR-3, and was cloned from a PC-3 cDNA library. Its isolation and characteristics are described in detail in Joukov *et al.*, EMBO J., 1996 15 290-298.

[0013] VEGF-D was isolated from a human breast cDNA library, commercially available from Clontech, by screening with an expressed sequence tag obtained from a human cDNA library designated "Soares Breast 3NbHBst" as a hybridization probe (Achen *et al.*, Proc. Natl. Acad. Sci. USA, 1998 95 548-553). Its isolation and characteristics are described in detail in International Patent Application No. PCT/US97/14696 (WO98/07832).

[0014] The VEGF-D gene is broadly expressed in the adult human, but is certainly not ubiquitously expressed. VEGF-D is strongly expressed in heart, lung and skeletal muscle. Intermediate levels of VEGF-D are expressed in spleen, ovary, small intestine and colon, and a lower expression occurs in kidney,

pancreas, thymus, prostate and testis. No VEGF-D mRNA was detected in RNA from brain, placenta, liver or peripheral blood leukocytes.

[0015] PlGF was isolated from a term placenta cDNA library. Its isolation and characteristics are described in detail in Maglione *et al.*, Proc. Natl. Acad. Sci. USA, 1991 88 9267-9271. Presently its biological function is not well understood.

[0016] VEGF3 was isolated from a cDNA library derived from colon tissue. VEGF3 is stated to have about 36% identity and 66% similarity to VEGF. The method of isolation of the gene encoding VEGF3 is unclear and no characterization of the biological activity is disclosed.

[0017] Similarity between two proteins is determined by comparing the amino acid sequence and conserved amino acid substitutions of one of the proteins to the sequence of the second protein, whereas identity is determined without including the conserved amino acid substitutions.

[0018] PDGF/VEGF family members act primarily by binding to receptor tyrosine kinases. Five endothelial cell-specific receptor tyrosine kinases have been identified, namely VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), VEGFR-3 (Flt4), Tie and Tek/Tie-2. All of these have the intrinsic tyrosine kinase activity which is necessary for signal transduction. The essential, specific role in vasculogenesis and angiogenesis of VEGFR-1, VEGFR-2, VEGFR-3, Tie and

Tek/Tie-2 has been demonstrated by targeted mutations inactivating these receptors in mouse embryos.

[0019] The only receptor tyrosine kinases known to bind VEGFs are VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR-1 and VEGFR-2 bind VEGF with high affinity, and VEGFR-1 also binds VEGF-B and PlGF. VEGF-C has been shown to be the ligand for VEGFR-3, and it also activates VEGFR-2 (Joukov *et al.*, The EMBO Journal, 1996 15 290-298). VEGF-D binds to both VEGFR-2 and VEGFR-3. A ligand for Tek/Tie-2 has been described in International Patent Application No. PCT/US95/12935 (WO 96/11269) by Regeneron Pharmaceuticals, Inc. The ligand for Tie has not yet been identified.

[0020] Recently, a novel 130-135 kDa VEGF isoform specific receptor has been purified and cloned (Soker *et al.*, Cell, 1998 92 735-745). The VEGF receptor was found to specifically bind the $VEGF_{165}$ isoform via the exon 7 encoded sequence, which shows weak affinity for heparin (Soker *et al.*, Cell, 1998 92 735-745). Surprisingly, the receptor was shown to be identical to human neuropilin-1 (NP-1), a receptor involved in early stage neuromorphogenesis. PlGF-2 also appears to interact with NP-1 (Migdal *et al.*, J. Biol. Chem., 1998 273 22272-22278).

[0021] VEGFR-1, VEGFR-2 and VEGFR-3 are expressed differently by endothelial cells. Both VEGFR-1 and VEGFR-2 are expressed in blood vessel endothelia (Oelrichs *et al.*, Oncogene, 1992 8 11-18; Kaipainen *et al.*, J. Exp.

Med., 1993 178 2077-2088; Dumont *et al.*, Dev. Dyn., 1995 203 80-92; Fong *et al.*, Dev. Dyn., 1996 207 1-10) and VEGFR-3 is mostly expressed in the lymphatic endothelium of adult tissues (Kaipainen *et al.*, Proc. Natl. Acad. Sci. USA, 1995 9 3566-3570). VEGFR-3 is also expressed in the blood vasculature surrounding tumors.

[0022] Disruption of the VEGFR genes results in aberrant development of the vasculature leading to embryonic lethality around midgestation. Analysis of embryos carrying a completely inactivated VEGFR-1 gene suggests that this receptor is required for functional organization of the endothelium (Fong *et al.*, Nature, 1995 376 66-70). However, deletion of the intracellular tyrosine kinase domain of VEGFR-1 generates viable mice with a normal vasculature (Hiratsuka *et al.*, Proc. Natl. Acad. Sci. USA 1998 95 9349-9354). The reasons underlying these differences remain to be explained but suggest that receptor signalling via the tyrosine kinase is not required for the proper function of VEGFR-1. Analysis of homozygous mice with inactivated alleles of VEGFR-2 suggests that this receptor is required for endothelial cell proliferation, hematopoesis and vasculogenesis (Shalaby *et al.*, Nature, 1995 376 62-66; Shalaby *et al.*, Cell, 1997 89 981-990). Inactivation of VEGFR-3 results in cardiovascular failure due to abnormal organization of the large vessels (Dumont *et al.* Science, 1998 282 946-949).

[0023] Although VEGFR-1 is mainly expressed in endothelial cells during development, it can also be found in hematopoetic precursor cells during early stages of embryogenesis (Fong *et al.*, Nature, 1995 376 66-70). It is also is expressed by most, if not all, vessels in embryos (Breier *et al.*, Dev. Dyn., 1995 204 228-239; Fong *et al.*, Dev. Dyn., 1996 207 1-10). In adults, monocytes and macrophages also express this receptor (Barleon *et al.*, Blood, 1996 87 3336-3343).

[0024] The receptor VEGFR-3 is widely expressed on endothelial cells during early embryonic development, but as embryogenesis proceeds, it becomes restricted to venous endothelium and then to the lymphatic endothelium (Kaipainen *et al.*, Cancer Res., 1994 54 6571-6577; Kaipainen *et al.*, Proc. Natl. Acad. Sci. USA, 1995 92 3566-3570). VEGFR-3 continues to be expressed on lymphatic endothelial cells in adults. This receptor is essential for vascular development during embryogenesis. Targeted inactivation of both copies of the VEGFR-3 gene in mice resulted in defective blood vessel formation characterized by abnormally organized large vessels with defective lumens, leading to fluid accumulation in the pericardial cavity and cardiovascular failure at post-coital day 9.5. On the basis of these findings it has been proposed that VEGFR-3 is required for the maturation of primary vascular networks into larger blood vessels. However, the role of VEGFR-3 in the development of the lymphatic vasculature could not be studied in these mice because the embryos died before the lymphatic system emerged. Nevertheless it is assumed that VEGFR-3 plays

a role in development of the lymphatic vasculature and lymphangiogenesis given its specific expression in lymphatic endothelial cells during embryogenesis and adult life. This is supported by the finding that ectopic expression of VEGF-C, a ligand for VEGFR- 3, in the skin of transgenic mice, resulted in lymphatic endothelial cell proliferation and vessel enlargement in the dermis. Furthermore this suggests that VEGF-C may have a primary function in lymphatic endothelium, and a secondary function in angiogenesis and permeability regulation which is shared with VEGF (Joukov *et al.*, EMBO J., 1996 15 290-298).

[0025] Some inhibitors of the VEGF/VEGF-receptor system have been shown to prevent tumor growth via an anti-angiogenic mechanism; see Kim *et al.*, Nature, 1993 362 841-844 and Saleh *et al.*, Cancer Res., 1996 56 393-401.

[0026] As mentioned above, the VEGF family of growth factors are members of the PDGF family. PDGF plays a important role in the growth and/or motility of connective tissue cells, fibroblasts, myofibroblasts and glial cells (Heldin *et al.*, "Structure of platelet-derived growth factor: Implications for functional properties", Growth Factor, 1993 8 245-252). In adults, PDGF stimulates wound healing (Robson *et al.*, Lancet, 1992 339 23-25). Structurally, PDGF isoforms are disulfide-bonded dimers of homologous A- and B-polypeptide chains, arranged as homodimers (PDGF-AA and PDGF-BB) or a heterodimer (PDGF-AB).

[0027] PDGF isoforms exert their effects on target cells by binding to two structurally related receptor tyrosine kinases (RTKs). The alpha-receptor binds both the A- and B-chains of PDGF, whereas the beta-receptor binds only the B-chain. These two receptors are expressed by many *in vitro* grown cell lines, and are mainly expressed by mesenchymal cells *in vivo*. The PDGFs regulate cell proliferation, cell survival and chemotaxis of many cell types in vitro (reviewed in Heldin *et al.*, Biochim Biophys Acta., 1998 1378 F79-113). In vivo, they exert their effects in a paracrine mode since they often are expressed in epithelial (PDGF-A) or endothelial cells (PDGF-B) in close apposition to the PDGFR expressing mesenchyme. In tumor cells and in cell lines grown *in vitro*, coexpression of the PDGFs and the receptors generate autocrine loops which are important for cellular transformation (Betsholtz *et al.*, Cell, 1984 39 447-57; Keating *et al.*, J. R. Coll Surg Edinb., 1990 35 172-4). Overexpression of the PDGFs have been observed in several pathological conditions, including malignancies, arteriosclerosis, and fibroproliferative diseases (reviewed in Heldin *et al.*, The Molecular and Cellular Biology of Wound Repair, New York: Plenum Press, 1996, 249-273).

[0028] The importance of the PDGFs as regulators of cell proliferation and survival are well illustrated by recent gene targeting studies in mice that have shown distinct physiological roles for the PDGFs and their receptors despite the overlapping ligand specificities of the PDGFRs. Homozygous null mutations for either of the two PDGF ligands or the receptors are lethal. Approximately 50%

of the homozygous PDGF-A deficient mice have an early lethal phenotype, while the surviving animals have a complex postnatal phenotype with lung emphysema due to improper alveolar septum formation because of a lack of alveolar myofibroblasts (Boström *et al.*, Cell, 1996 85 863-873). The PDGF-A deficient mice also have a dermal phenotype characterized by thin dermis, misshapen hair follicles and thin hair (Karlsson *et al.*, Development, 1999 126 2611-2). PDGF-A is also required for normal development of oligodendrocytes and subsequent myelination of the central nervous system (Fruttiger *et al.*, Development, 1999 126 457-67). The phenotype of PDGFR-alpha deficient mice is more severe with early embryonic death at E10, incomplete cephalic closure, impaired neural crest development, cardiovascular defects, skeletal defects, and edemas (Soriano *et al.*, Development, 1997 124 2691-70). The PDGF-B and PDGFR-beta deficient mice develop similar phenotypes that are characterized by renal, hematological and cardiovascular abnormalities (Levéen *et al.*, Genes Dev., 1994 8 1875-1887; Soriano *et al.*, Genes Dev., 1994 8 1888-96; Lindahl *et al.*, Science, 1997 277 242-5; Lindahl, Development, 1998 125 3313-2), where the renal and cardiovascular defects, at least in part, are due to the lack of proper recruitment of mural cells (vascular smooth muscle cells, pericytes or mesangial cells) to blood vessels (Levéen *et al.*, Genes Dev., 1994 8 1875-1887; Lindahl *et al.*, Science, 1997 277 242-5; Lindahl *et al.*, Development, 1998 125 3313-2).

[0029] Most recently, an additional member of the PDGF/VEGF family of growth factors was identified, PDGF-C. PDGF-C is described in International

Patent Application PCT/US99/22668 (WO 00/18212), filed September 30, 1999. PDGF-C has a two-domain structure not previously recognized within this family of growth factors, a N-terminal C1r/C1s/embryonic sea urchin protein Uegf/bone morphogenetic protein 1 (CUB) domain, and a C-terminal PDGF/VEGF homology domain (P/VHD). The structure of the P/VHD in PDGF-C shows a low overall sequence identity with other PDGF/VEGF homology domains, although the eight invariant cysteine residues involved in inter- and intra-molecular disulfide bond formation are present. The cysteine spacing in the central, most conserved region of this domain is different from other PDGF/VEGF domains, with an insertion of three amino acid residues. Despite the fact that the insertion occurs close to the loop 2 region which has been proposed to be involved in receptor binding, it was shown that this domain of PDGF-CC binds PDGFR-alpha with almost identical affinities as homodimers of PDGF-A or -B chains. In addition, four extra cysteine residues are present in this domain. Full length and truncated PDGF-CC was found not to bind to VEGFR-1, -2 or -3, or to PDGFR-beta.

[0030] PDGF-C requires proteolytic removal of the N-terminal CUB domain for receptor binding and activation of the receptor. This indicates that the CUB domains are likely to sterically block the receptor binding epitopes of the unprocessed dimer. The *in vitro* and *in vivo* proteolytically processed proteins are devoid of N-terminal portions corresponding to more than 14-16 kDa as determined from SDS-PAGE analysis which is consistent with a loss of the 110

amino acid long CUB domain and a part of the hinge region between the CUB and core domains that vary in length.

[0031] PDGF-C is not proteolytically processed during secretion in transfected COS cells indicating that proteolytic removal of the CUB domain occurs extracellularly, and not during secretion. This is in contrast to PDGF-A and -B (Östman *et al.*, J. Cell. Biol., 1992 118 509-519) which appear to be processed intracellularly by furin-like endoproteases (Nakayama *et al.*, Biochem J., 1997 327 625-635).

[0032] Northern blots show PDGF-C mRNA in a variety of human tissues, including heart, liver, kidney, pancreas and ovary.

[0033] *In situ* localization studies demonstrate expression of PDGF-C in certain epithelial structures, and PDGFR-alpha in adjacent mesenchyme, indicating the potential of paracrine signaling in the developing embryo. PDGF-C expression seems particularly abundant at sites of ongoing ductal morphogenesis, indicating a role of the factor in connective tissue remodeling at these sites. The expression pattern is distinct from that of PDGF-A or PDGF-B indicating that the three growth factors have different roles despite their similar PDGFR-alpha binding and signaling activities. This is illustrated by the mouse embryonic kidney, in which PDGF-C is expressed in early aggregates of metanephric mesenchyme undergoing epithelial conversion, whereas PDGF-A is expressed in more mature tubular structures, and PDGF-B by vascular

endothelial cells. PDGFR-alpha is expressed in the mesenchyme of the kidney cortex, adjacent to the sites of PDGF-C expression, indicating that this mesenchyme may be targeted specifically by PDGF-C. Indeed, PDGFR-alpha -/- mouse embryos show an extensive loss of the cortical mesenchyme adjacent to sites of PDGF-C expression, not seen in PDGF-A -/- mice or in PDGF-A/B -/- mice, indicating that PDGF-C has an essential role in the development of kidney mesenchyme.

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SUMMARY OF THE INVENTION

[0034] The invention generally provides an isolated novel growth factor, PDGF-D, a polypeptide that has the ability to stimulate, or enhance, or both, one or more of proliferation, differentiation, growth, and motility of cells expressing a PDGF-D receptor. The cells affected by the inventive growth factor include, but are not limited to, endothelial cells, connective tissue cells, myofibroblasts and glial cells. The invention also provides isolated polynucleotide molecules encoding the novel growth factor, and compositions useful for diagnostic and/or therapeutic applications.

[0035] According to one aspect, the invention provides an isolated nucleic acid molecule which comprises a polynucleotide sequence having at least 85% identity, more preferably at least 90%, and still more preferably at least 95% identity, and most preferably at 100% identity to at least nucleotides 1 to 600 of the sequence set out SEQ ID NO:3, at least nucleotides 1 to 966 of the sequence

set out in SEQ ID NO:5, at least nucleotides 173 to 1288 of the sequence set out in SEQ ID NO:7 at least nucleotides 938 to 1288 set out in SEQ ID NO:7, at least nucleotides 1 to 1110 of SEQ ID NO:35, at least nucleotides 1-1092 of SEQ ID NO:37, or SEQ ID NO:39. The sequence of at least nucleotides 1 to 600 of the sequence set out in Figure 3 (SEQ ID NO:3) or at least nucleotides 1 to 966 of the sequence set out in Figure 5 (SEQ ID NO:5) encodes a 5'-truncated polypeptide, designated PDGF-D (formally designated "VEGF-G"), while at least nucleotides 173 to 1288 of the sequence set out in Figure 7 (SEQ ID NO:7) encodes a full-length PDGF-D. The sequence of at least nucleotides 1 to 1110 of SEQ ID NO:35 encodes a murine PDGF-D, while the sequence of at least nucleotides 1-1092 of SEQ ID NO:37 encodes an identical protein as SEQ ID NO:35 except for a six amino acid residue gap (a.a. #42-47) from the region between the signal sequence and the CUB domain (see below for details) , and SEQ ID NO:39 a C-terminal truncated protein of the polypeptide encoded by SEQ ID NO:35. The PDGF-D polynucleotide of the invention can be a naked plynucleotide and/or in a vector or liposome.

[0036] PDGF-D is structurally homologous to PDGF-A, PDGF-B, VEGF, VEGF-B, VEGF-C and VEGF-D. The sequence of at least nucleotides 938 to 1288 set out in Figure 7 (SEQ ID NO:7) encodes a portion of the PDGF/VEGF homology domain, which is the bioactive fragment of PDGF-D. This bioactive fragment would also be encoded by the sequence of at least nucleotides 1 to 600

of the sequence set out in Figure 3 (SEQ ID NO:3) or at least nucleotides 1 to 966 of the sequence set out in Figure 5 (SEQ ID NO:5).

[0037] According to a second aspect, the PDGF-D polypeptide of the invention has the ability to stimulate and/or enhance proliferation and/or differentiation and/or growth and/or motility of cells expressing a PDGF-D receptor including, but not limited to, endothelial cells, connective tissue cells, myofibroblasts and glial cells and comprises a sequence of amino acids having at least 85% identity, more preferably at least 90%, and still more preferably at least 95% identity, and most preferably at 100% identity to the amino acid sequence set out in SEQ ID NOs:4, 6, 8, 36, 38 or 40, or a fragment or analog thereof which has PDGF-D activity.

[0038] A preferred fragment is a truncated form of PDGF-D comprising a portion of the PDGF/VEGF homology domain (PVHD) of PDGF-D. The portion of the PVHD is from residues 255-371 of Figure 8 (SEQ ID NO:8) where the putative proteolytic processing site RKSK starts at amino acid residue 255 (SEQ ID NO:8). However, the PVHD extends toward the N terminus up to residue 235 of Figure 8 (SEQ ID NO:8). Herein the PVHD is defined as truncated PDGF-D. The truncated PDGF-D is the putative activated form of PDGF-D.

[0039] Another preferred fragment is a truncated form of PDGF-D comprising only the CUB domain, as exemplified by the sequence set forth in SEQ ID NO:40. There may exist PDGF-D receptors, other than PDGFR-beta,

that bind to the unprocessed or un-cleaved factor (CUB + PDGF-homology domain). The CUB domain alone may bind to these receptors and would prevent activation of said receptors by blocking the receptors from binding to un-cleaved factors.

[0040] As used in this application, percent sequence identity is determined by using the alignment tool of "MEGALIGN" from the Lasergene package (DNASTAR, Ltd. Abacus House, Manor Road, West Ealing, London W130AS United Kingdom). The MEGALIGN is based on the J. Hein method (Methods in Enzymology, 1990 183 626-645). The PAM 250 residue weight table is used with a gap penalty of eleven and a gap length penalty of three and a K-tuple value of two in the pairwise alignments. The alignment is then refined manually, and the number of identities are estimated in the regions available for a comparison.

[0041] Preferably the polypeptide has the ability to stimulate one or more of proliferation, differentiation, motility, survival or vascular permeability of cells expressing a PDGF-D receptor including, but not limited to, vascular endothelial cells, lymphatic endothelial cells, connective tissue cells (such as fibroblasts), myofibroblasts and glial cells. Preferably the polypeptide has the ability to stimulate wound healing. PDGF-D also has antagonistic effects on cells. For example, an antagonistic PDGF-D variant would be a partial PDGF-D molecule containing one intact full-length chain and one processed chain as a disulphide-linked dimer. In principle such a molecule would be monovalent and bind to

single PDGFR-beta receptors, but prevent their dimerization thereby blocking signal transduction. These antagonistic activities are also included in the biological activities of PDGF-D. Collectively, both the stimulating and antagonistic abilities are referred to hereinafter as "biological activities of PDGF-D" and can be readily tested by methods known in the art.

[0042] In another preferred aspect, the invention provides a polypeptide comprising an amino acid sequence:

PXCLLVXRCGGNCGC (SEQ ID NO:25)

which is unique to PDGF-D and differs from the other members of the PDGF/VEGF family of growth factors because of the insertion of the three amino acid residues (NCG) between the third and fourth cysteines (see Figure 9).

[0043] Polypeptides comprising conservative substitutions, insertions, or deletions, but which still retain a biological activity of PDGF-D are within the scope of the invention. Persons skilled in the art will be well aware of methods which can readily be used to generate such polypeptides, for example the use of site-directed mutagenesis, or specific enzymatic cleavage and ligation. The skilled person will also be aware that peptidomimetic compounds or compounds in which one or more amino acid residues are replaced by a non-naturally occurring amino acid or an amino acid analog may retain the required aspects of the biological activity of PDGF-D. Such compounds can readily be made and tested for their ability to show the biological activity of PDGF-D by routine

activity assay procedures such as the fibroblast proliferation assay and are also within the scope of the invention.

[0044] In addition, possible variant forms of the PDGF-D polypeptide which may result from alternative splicing, as are known to occur with VEGF and VEGF-B, and naturally-occurring allelic variants of the nucleic acid sequence encoding PDGF-D are within the scope of the invention. Examples of such a variant include the polypeptides set forth in SEQ ID NOs: 38 and 40. Allelic variants are well known in the art, and represent alternative forms or a nucleic acid sequence which comprise substitution, deletion or addition of one or more nucleotides, but which do not result in any substantial functional alteration of the encoded polypeptide.

[0045] Such variant forms of PDGF-D can be prepared by targeting non-essential regions of the PDGF-D polypeptide for modification. These non-essential regions are expected to fall outside the strongly-conserved regions indicated in Figure 9 (SEQ ID NOs:8 and 32). In particular, the growth factors of the PDGF family, including PDGF-D, are dimeric. PDGF-D differs slightly from VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF, PDGF-A and PDGF-B because it shows complete conservation of only seven of the eight cysteine residues in the PVHD (Olofsson *et al.*, Proc. Natl. Acad. Sci. USA, 1996 93 2576-2581; Joukov *et al.*, EMBO J., 1996 15 290-298). These cysteines are thought to be involved in intra- and inter-molecular disulfide bonding. Loops 1, 2 and 3 of each subunit,

which are formed by intra-molecular disulfide bonding, are involved in binding to the receptors for the PDGF/VEGF family of growth factors (Andersson *et al.*, Growth Factors, 1995 12 159-164).

[0046] Persons skilled in the art thus are well aware that these cysteine residues generally should be preserved and that the active sites present in loops 1, 2 and 3 also should be preserved. However, other regions of the molecule can be expected to be of lesser importance for biological function, and therefore offer suitable targets for modification. Modified polypeptides can readily be tested for their ability to show the biological activity of PDGF-D by routine activity assay procedures such as the fibroblast proliferation assay.

[0047] It is contemplated that some modified PDGF-D polypeptides will have the ability to bind to PDGF-D receptors on cells including, but not limited to, endothelial cells, connective tissue cells, myofibroblasts and/or glial cells, but will be unable to stimulate cell proliferation, differentiation, migration, motility or survival or to induce vascular proliferation, connective tissue development or wound healing. These modified polypeptides are expected to be able to act as competitive or non-competitive inhibitors of the PDGF-D polypeptides and growth factors of the PDGF/VEGF family, and to be useful in situations where prevention or reduction of the PDGF-D polypeptide or PDGF/VEGF family growth factor action is desirable. Thus such receptor-binding but non-mitogenic, non-differentiation inducing, non-migration inducing, non-motility inducing,

non-survival promoting, non-connective tissue promoting, non-wound healing or non-vascular proliferation inducing variants of the PDGF-D polypeptide are also within the scope of the invention, and are referred to herein as "receptor-binding but otherwise inactive variants." Because PDGF-D forms a dimer in order to activate its only known receptor, it is contemplated that one monomer comprises the receptor-binding but otherwise inactive variant modified PDGF-D polypeptide and a second monomer comprises a wild-type PDGF-D or a wild-type growth factor of the PDGF/VEGF family. These dimers can bind to its corresponding receptor but cannot induce downstream signaling.

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[0048] It is also contemplated that there are other modified PDGF-D polypeptides that can prevent binding of a wild-type PDGF-D or a wild-type growth factor of the PDGF/VEGF family to its corresponding receptor on cells including, but not limited to, endothelial cells, connective tissue cells (such as fibroblasts), myofibroblasts and/or glial cells. Thus these dimers will be unable to stimulate endothelial cell proliferation, differentiation, migration, survival, or induce vascular permeability, and/or stimulate proliferation and/or differentiation and/or motility of connective tissue cells, myofibroblasts or glial cells. These modified polypeptides are expected to be able to act as competitive or non-competitive inhibitors of the PDGF-D growth factor or a growth factor of the PDGF/VEGF family, and to be useful in situations where prevention or reduction of the PDGF-D growth factor or PDGF/VEGF family growth factor action is desirable. Such situations include the tissue remodeling that takes

place during invasion of tumor cells into a normal cell population by primary or metastatic tumor formation. Thus such PDGF-D or PDGF/VEGF family growth factor-binding but non-mitogenic, non-differentiation inducing, non-migration inducing, non-motility inducing, non-survival promoting, non-connective tissue promoting, non-wound healing or non-vascular proliferation inducing variants of the PDGF-D growth factor are also within the scope of the invention, and are referred to herein as "the PDGF-D growth factor-dimer forming but otherwise inactive or interfering variants."

[0049] An example of a PDGF-D growth factor-dimer forming but otherwise inactive or interfering variant is where the PDGF-D has a mutation which prevents cleavage of CUB domain from the protein. It is further contemplated that a PDGF-D growth factor-dimer forming but otherwise inactive or interfering variant could be made to comprise a monomer, preferably a monomer whose own N-terminal CUB domain has been removed (hereinafter a "CUB-removed monomer") of VEGF, VEGF-B, VEGF-C, VEGF-D, PDGF-C, PDGF-A, PDGF-B, PDGF-C, PDGF-D or PlGF linked to a CUB domain that has a mutation which prevents cleavage of CUB domain from the protein. Dimers formed with the above mentioned PDGF-D growth factor-dimer forming but otherwise inactive or interfering variants and the monomers linked to the mutant CUB domain would be unable to bind to their corresponding receptors.

[0050] A variation on this contemplation would be to insert a proteolytic site between a CUB-removed monomer of VEGF, VEGF-B, VEGF-C, VEGF-D, PDGF-C, PDGF-A, PDGF-B, PDGF-C, PDGF-D or PlGF and the mutant CUB domain which is dimerized to a CUB-removed monomer of VEGF, VEGF-B, VEGF-C, VEGF-D, PDGF-A, PDGF-B, PDGF-C, PDGF-D or PlGF. Addition of the specific protease(s) for this proteolytic site would cleave the CUB domain and thereby release an activated dimer that can then bind to its corresponding receptor. In this way, a controlled release of an activated dimer is made possible.

[0051] According to a third aspect, the invention provides a purified and isolated nucleic acid encoding a polypeptide or polypeptide fragment of the invention as defined above. The nucleic acid may be DNA, genomic DNA, cDNA or RNA, and may be single-stranded or double stranded. The nucleic acid may be isolated from a cell or tissue source, or of recombinant or synthetic origin. Because of the degeneracy of the genetic code, the person skilled in the art will appreciate that many such coding sequences are possible, where each sequence encodes the amino acid sequence shown in Figure 4 (SEQ ID NO:4), Figure 6 (SEQ ID NO:6) or Figure 8 (SEQ ID NO:8), a bioactive fragment or analog thereof, a receptor-binding but otherwise inactive or partially inactive variant thereof or a PDGF-D dimer-forming but otherwise inactive or interfering variants thereof.

[0052] A fourth aspect of the invention provides vectors comprising the cDNA of the invention or a nucleic acid molecule according to the third aspect of the invention, and host cells transformed or transfected with nucleic acids molecules or vectors of the invention. These may be eukaryotic or prokaryotic in origin. These cells are particularly suitable for expression of the polypeptide of the invention, and include insect cells such as Sf9 cells, obtainable from the American Type Culture Collection (ATCC SRL-171), transformed with a baculovirus vector, and the human embryo kidney cell line 293-EBNA transfected by a suitable expression plasmid. Preferred vectors of the invention are expression vectors in which a nucleic acid according to the invention is operatively connected to one or more appropriate promoters and/or other control sequences, such that appropriate host cells transformed or transfected with the vectors are capable of expressing the polypeptide of the invention. Other preferred vectors are those suitable for transfection of mammalian cells, or for gene therapy, such as adenoviral-, vaccinia- or retroviral-based vectors or liposomes. A variety of such vectors is known in the art.

[0053] The invention also provides a method of making a vector capable of expressing a polypeptide encoded by a nucleic acid molecule according to the invention, comprising the steps of operatively connecting the nucleic acid molecule to one or more appropriate promoters and/or other control sequences, as described above.

[0054] The invention further provides a method of making a polypeptide according to the invention, comprising the steps of expressing a nucleic acid or vector of the invention in a host cell, and isolating the polypeptide from the host cell or from the host cell's growth medium.

[0055] In yet a further aspect, the invention provides an antibody specifically reactive with a polypeptide of the invention or a fragment of the polypeptide. This aspect of the invention includes antibodies specific for the variant forms, immunoreactive fragments, analogs and recombinants of PDGF-D. Such antibodies are useful as inhibitors or antagonists of PDGF-D and as diagnostic agents for detecting and quantifying PDGF-D. Polyclonal or monoclonal antibodies may be used. Monoclonal and polyclonal antibodies can be raised against polypeptides of the invention or fragment or analog thereof using standard methods in the art. In addition the polypeptide can be linked to an epitope tag, such as the FLAG® octapeptide (Sigma, St. Louis, MO), to assist in affinity purification. For some purposes, for example where a monoclonal antibody is to be used to inhibit effects of PDGF-D in a clinical situation, it may be desirable to use humanized or chimeric monoclonal antibodies. Such antibodies may be further modified by addition of cytotoxic or cytostatic drug(s). Methods for producing these, including recombinant DNA methods, are also well known in the art.

[0056] This aspect of the invention also includes an antibody which recognizes PDGF-D and is suitably labeled.

[0057] Polypeptides or antibodies according to the invention may be labeled with a detectable label, and utilized for diagnostic purposes. Similarly, the thus-labeled polypeptide of the invention may be used to identify its corresponding receptor *in situ*. The polypeptide or antibody may be covalently or non-covalently coupled to a suitable supermagnetic, paramagnetic, electron dense, ecogenic or radioactive agent for imaging. For use in diagnostic assays, radioactive or non-radioactive labels may be used. Examples of radioactive labels include a radioactive atom or group, such as ^{125}I or ^{32}P. Examples of non-radioactive labels include enzymatic labels, such as horseradish peroxidase or fluorimetric labels, such as fluorescein-5-isothiocyanate (FITC). Labeling may be direct or indirect, covalent or non-covalent.

[0058] Clinical applications of the invention include diagnostic applications, acceleration of angiogenesis in tissue or organ transplantation, or stimulation of wound healing, or connective tissue development, or to establish collateral circulation in tissue infarction or arterial stenosis, such as coronary artery disease, and inhibition of angiogenesis in the treatment of cancer or of diabetic retinopathy and inhibition of tissue remodeling that takes place during invasion of tumor cells into a normal cell population by primary or metastatic tumor

formation. Quantitation of PDGF-D in cancer biopsy specimens may be useful as an indicator of future metastatic risk.

[0059] PDGF-D may also be relevant to a variety of lung conditions. PDGF-D assays could be used in the diagnosis of various lung disorders. PDGF-D could also be used in the treatment of lung disorders to improve blood circulation in the lung and/or gaseous exchange between the lungs and the blood stream. Similarly, PDGF-D could be used to improve blood circulation to the heart and O_2 gas permeability in cases of cardiac insufficiency. In a like manner, PDGF-D could be used to improve blood flow and gaseous exchange in chronic obstructive airway diseases.

[0060] Thus the invention provides a method of stimulation of angiogenesis, lymphangiogenesis, neovascularization, connective tissue development and/or wound healing in a mammal in need of such treatment, comprising the step of administering an effective dose of PDGF-D, or a fragment or an analog thereof which has the biological activity of PDGF-D to the mammal. Optionally the PDGF-D, or fragment or analog thereof may be administered together with, or in conjunction with, one or more of VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF, PDGF-A, PDGF-B, PDGF-C, FGF and/or heparin.

[0061] Conversely, PDGF-D antagonists (e.g. antibodies and/or competitive or noncompetitive inhibitors of binding of PDGF-D in both dimer formation and receptor binding) could be used to treat conditions, such as congestive heart

failure, involving accumulation of fluid in, for example, the lung resulting from increases in vascular permeability, by exerting an offsetting effect on vascular permeability in order to counteract the fluid accumulation. Administrations of PDGF-D could be used to treat malabsorptive syndromes in the intestinal tract, liver or kidneys as a result of its blood circulation increasing and vascular permeability increasing activities.

[0062] Thus, the invention provides a method of inhibiting angiogenesis, lymphangiogenesis, neovascularization, connective tissue development and/or wound healing in a mammal in need of such treatment, comprising the step of administering an effective amount of an antagonist of PDGF-D to the mammal. The antagonist may be any agent that prevents the action of PDGF-D, either by preventing the binding of PDGF-D to its corresponding receptor on the target cell, or by preventing activation of the receptor, such as using receptor-binding PDGF-D variants. Suitable antagonists include, but are not limited to, antibodies directed against PDGF-D; competitive or non-competitive inhibitors of binding of PDGF-D to the PDGF-D receptor(s), such as the receptor-binding or PDGF-D dimer-forming but non-mitogenic PDGF-D variants referred to above; and anti-sense nucleotide sequences as described below.

[0063] A method is provided for determining agents that bind to an activated truncated form of PDGF-D. The method comprises contacting an activated truncated form of PDGF-D with a test agent and monitoring binding by

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any suitable means. Potential binding agents include proteins and other substances.The invention provides a screening system for discovering agents that bind an activated truncated form of PDGF-D. The screening system comprises preparing an activated truncated form of PDGF-D, exposing the activated truncated form of PDGF-D to a test agent, and quantifying the binding of said agent to the activated truncated form of PDGF-D by any suitable means. The inhibitory effects of a binding agent are further determined by assaying the PDGF-D activities of the PDGF-D polypeptides bound with the binding agent. Both in vivo and in vitro assay methods may be used. Specifically, this screening system is used to identify agents which inhibit the proteolytic cleavage of the full length PDGF-D protein and thereby prevent the release of the activated truncated form of PDGF-D. For this use, the full length PDGF-D is generally preferred.

[0064] Use of this screen system provides a means to determine compounds that may alter the biological function of PDGF-D. This screening method may be adapted to large-scale, automated procedures such as a PANDEX® (Baxter-Dade Diagnostics) system, allowing for efficient high-volume screening of potential therapeutic agents.

[0065] For this screening system, an activated truncated form of PDGF-D or full length PDGF-D is prepared as described herein, preferably using recombinant DNA technology. A test agent, e.g. a compound or protein, is

introduced into a reaction vessel containing the activated truncated form of or full length PDGF-D. Binding of the test agent to the activated truncated form of or full length PDGF-D is determined by any suitable means which include, but is not limited to, radioactively- or chemically-labeling the test agent. Binding of the activated truncated form of or full length PDGF-D may also be carried out by a method disclosed in U.S. Patent 5,585,277, which is incorporated by reference. In this method, binding of the test agent to the activated truncated form of or full length PDGF-D is assessed by monitoring the ratio of folded protein to unfolded protein. Examples of this monitoring can include, but are not limited to, monitoring the sensitivity of the activated truncated form of or full length PDGF-D to a protease, or amenability to binding of the protein by a specific antibody against the folded state of the protein.

[0066] Those of skill in the art will recognize that IC_{50} values are dependent on the selectivity of the agent tested. For example, an agent with an IC_{50} which is less than 10 nM is generally considered an excellent candidate for drug therapy. However, an agent which has a lower affinity, but is selective for a particular target, may be an even better candidate. Those skilled in the art will recognize that any information regarding the binding potential, inhibitory activity or selectivity of a particular agent is useful toward the development of pharmaceutical products.

[0067] Where PDGF-D or a PDGF-D antagonist is to be used for therapeutic purposes, the dose(s) and route of administration will depend upon the nature of the patient and condition to be treated, and will be at the discretion of the attending physician or veterinarian. Suitable routes include oral, subcutaneous, intramuscular, intraperitoneal or intravenous injection, parenteral, topical application, implants *etc.* Topical application of PDGF-D may be used in a manner analogous to VEGF. Where used for wound healing or other use in which enhanced angiogenesis is advantageous, an effective amount of the truncated active form of PDGF-D is administered to an organism in need thereof in a dose between about 0.1 and 1000 g/kg body weight.

[0068] The PDGF-D or a PDGF-D antagonist may be employed in combination with a suitable pharmaceutical carrier. The resulting compositions comprise a therapeutically effective amount of PDGF-D or a PDGF-D antagonist, and a pharmaceutically acceptable non-toxic salt thereof, and a pharmaceutically acceptable solid or liquid carrier or adjuvant. Examples of such a carrier or adjuvant include, but are not limited to, saline, buffered saline, Ringer's solution, mineral oil, talc, corn starch, gelatin, lactose, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride, alginic acid, dextrose, water, glycerol, ethanol, thickeners, stabilizers, suspending agents and combinations thereof. Such compositions may be in the form of solutions, suspensions, tablets, capsules, creams, salves, elixirs, syrups, wafers, ointments or other conventional forms. The formulation to suit the mode of administration.

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Compositions which comprise PDGF-D may optionally further comprise one or more of PDGF-A, PDGF-B, PDGF-C, VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF and/or heparin. Compositions comprising PDGF-D will contain from about 0.1% to 90% by weight of the active compound(s), and most generally from about 10% to 30%.

[0069] For intramuscular preparations, a sterile formulation, preferably a suitable soluble salt form of the truncated active form of PDGF-D, such as hydrochloride salt, can be dissolved and administered in a pharmaceutical diluent such as pyrogen-free water (distilled), physiological saline or 5% glucose solution. A suitable insoluble form of the compound may be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, e.g. an ester of a long chain fatty acid such as ethyl oleate.

[0070] According to yet a further aspect, the invention provides diagnostic/prognostic devices typically in the form of test kits. For example, in one embodiment of the invention there is provided a diagnostic/prognostic test kit comprising an antibody to PDGF-D and a means for detecting, and more preferably evaluating, binding between the antibody and PDGF-D. In one preferred embodiment of the diagnostic/prognostic device according to the invention, a second antibody (the secondary antibody) directed against antibodies of the same isotype and animal source of the antibody directed against PDGF-D (the primary antibody) is provided. The secondary antibody is

coupled directly or indirectly to a detectable label, and then either an unlabeled primary antibody or PDGF-D is substrate-bound so that the PDGF-D/primary antibody interaction can be established by determining the amount of label bound to the substrate following binding between the primary antibody and PDGF-D and the subsequent binding of the labeled secondary antibody to the primary antibody. In a particularly preferred embodiment of the invention, the diagnostic/prognostic device may be provided as a conventional enzyme-linked immunosorbent assay (ELISA) kit.

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[0071] In another alternative embodiment, a diagnostic/prognostic device may comprise polymerase chain reaction means for establishing sequence differences of a PDGF-D of a test individual and comparing this sequence structure with that disclosed in this application in order to detect any abnormalities, with a view to establishing whether any aberrations in PDGF-D expression are related to a given disease condition.

[0072] In addition, a diagnostic/prognostic device may comprise a restriction length polymorphism (RFLP)generating means utilizing restriction enzymes and genomic DNA from a test individual to generate a pattern of DNA bands on a gel and comparing this pattern with that disclosed in this application in order to detect any abnormalities, with a view to establishing whether any aberrations in PDGF-D expression are related to a given disease condition.

[0073] In accordance with a further aspect, the invention relates to a method of detecting aberrations in PDGF-D gene structure in a test subject which may be associated with a disease condition in the test subject. This method comprises providing a DNA sample from said test subject; contacting the DNA sample with a set of primers specific to PDGF-D DNA operatively coupled to a polymerase and selectively amplifying PDGF-D DNA from the sample by polymerase chain reaction, and comparing the nucleotide sequence of the amplified PDGF-D DNA from the sample with the nucleotide sequences shown in Figure 3 (SEQ ID NO:3), Figure 5 (SEQ ID NO:5) or Figure 7 (SEQ ID NO:7). The invention also includes the provision of a test kit comprising a pair of primers specific to PDGF-D DNA operatively coupled to a polymerase, whereby said polymerase is enabled to selectively amplify PDGF-D DNA from a DNA sample.

[0074] The invention also provides a method of detecting PDGF-D in a biological sample, comprising the step of contacting the sample with a reagent capable of binding PDGF-D, and detecting the binding. Preferably the reagent capable of binding PDGF-D is an antibody directed against PDGF-D, particularly a monoclonal antibody. In a preferred embodiment the binding and/or extent of binding is detected by means of a detectable label; suitable labels are discussed above.

[0075] In another aspect, the invention relates to a protein dimer comprising the PDGF-D polypeptide, particularly a disulfide-linked dimer. The protein dimers of the invention include both homodimers of PDGF-D polypeptide and heterodimers of PDGF-D and VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF, PDGF-A, PDGF-B or PDGF-C.

[0076] According to a yet further aspect of the invention there is provided a method for isolation of PDGF-D comprising the step of exposing a cell which expresses PDGF-D to heparin to facilitate release of PDGF-D from the cell, and purifying the thus-released PDGF-D.

[0077] Another aspect of the invention involves providing a vector comprising an anti-sense nucleotide sequence which is complementary to at least a part of a DNA sequence which encodes PDGF-D or a fragment or analog thereof that has the biological activity of PDGF-D. In addition the anti-sense nucleotide sequence can be to the promoter region of the PDGF-D gene or other non-coding region of the gene which may be used to inhibit, or at least mitigate, PDGF-D expression.

[0078] According to a yet further aspect of the invention such a vector comprising an anti-sense sequence may be used to inhibit, or at least mitigate, PDGF-D expression. The use of a vector of this type to inhibit PDGF-D expression is favored in instances where PDGF-D expression is associated with a disease, for example where tumors produce PDGF-D in order to provide for

angiogenesis, or tissue remodeling that takes place during invasion of tumor cells into a normal cell population by primary or metastatic tumor formation. Transformation of such tumor cells with a vector containing an anti-sense nucleotide sequence would suppress or retard angiogenesis, and so would inhibit or retard growth of the tumor or tissue remodeling.

[0079] Another aspect of the invention relates to the discovery that the full length PDGF-D protein is likely to be a latent growth factor that needs to be activated by proteolytic processing to release an active PDGF/VEGF homology domain. A putative proteolytic site is found in residues 255-258 in the full length protein, residues -RKSK- (SEQ ID NO:9). This is a dibasic motif. The -RKSK- (SEQ ID NO:9) putative proteolytic site is also found in PDGF-A, PDGF-B, VEGF-C and VEGF-D. In these four proteins, the putative proteolytic site is also found just before the minimal domain for the PDGF/VEGF homology domain. Together these facts indicate that this is the proteolytic site.

[0080] Preferred proteases include, but are not limited, to plasmin, Factor X and enterokinase. The N-terminal CUB domain may function as an inhibitory domain which might be used to keep PDGF-D in a latent form in some extracellular compartment and which is removed by limited proteolysis when PDGF-D is needed.

[0081] According to this aspect of the invention, a method is provided for producing an activated truncated form of PDGF-D or for regulating receptor-

binding specificity of PDGF-D. These methods comprise the steps of expressing an expression vector comprising a polynucleotide encoding a polypeptide having the biological activity of PDGF-D and supplying a proteolytic amount of at least one enzyme for processing the expressed polypeptide to generate the activated truncated form of PDGF-D.

[0082] This aspect also includes a method for selectively activating a polypeptide having a growth factor activity. This method comprises the step expressing an expression vector comprising a polynucleotide encoding a polypeptide having a growth factor activity, a CUB domain and a proteolytic site between the polypeptide and the CUB domain, and supplying a proteolytic amount of at least one enzyme for processing the expressed polypeptide to generate the activated polypeptide having a growth factor activity.

[0083] In addition, this aspect includes the isolation of a nucleic acid molecule which codes for a polypeptide having the biological activity of PDGF-D and a polypeptide thereof which comprises a proteolytic site having the amino acid sequence RKSR (SEQ ID NO:9) or a structurally conserved amino acid sequence thereof.

[0084] Also this aspect includes an isolated dimer comprising an activated monomer of PDGF-D and an activated monomer of VEGF, VEGF-B, VEGF-C, VEGF-D, PDGF-D, PDGF-A, PDGF-B, PDGF-C or PlGF linked to a CUB domain, or alternatively, an activated monomer of VEGF, VEGF-B, VEGF-C,

VEGF-D, PDGF-D, PDGF-A, PDGF-B or PlGF and an activated monomer of PDGF-D linked to a CUB domain. The isolated dimer may or may not include a proteolytic site between the activated monomer and the CUB domain.

[0085] Polynucleotides of the invention such as those described above, fragments of those polynucleotides, and variants of those polynucleotides with sufficient similarity to the non-coding strand of those polynucleotides to hybridize thereto under stringent conditions all are useful for identifying, purifying, and isolating polynucleotides encoding other, non-human, mammalian forms of PDGF-D. Thus, such polynucleotide fragments and variants are intended as aspects of the invention. Exemplary stringent hybridization conditions are as follows: hybridization at 42°C in 5X SSC, 20 mM $NaPO_4$, pH 6.8, 50% formamide; and washing at 42°C in 0.2X SSC. Those skilled in the art understand that it is desirable to vary these conditions empirically based on the length and the GC nucleotide base content of the sequences to be hybridized, and that formulas for determining such variation exist. See for example Sambrook *et al*, "Molecular Cloning: A Laboratory Manual", Second Edition, pages 9.47-9.51, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1989).

[0086] Moreover, purified and isolated polynucleotides encoding other, non-human, mammalian PDGF-D forms also are aspects of the invention, as are the polypeptides encoded thereby and antibodies that are specifically immunoreactive with the non-human PDGF-D variants. Thus, the invention

includes a purified and isolated mammalian PDGF-D polypeptide and also a purified and isolated polynucleotide encoding such a polypeptide.

[0087] It will be clearly understood that nucleic acids and polypeptides of the invention may be prepared by synthetic means or by recombinant means, or may be purified from natural sources.

[0088] It will be clearly understood that for the purposes of this specification the word "comprising" means "included but not limited to." The corresponding meaning applies to the word "comprises."

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BRIEF DESCRIPTION OF THE DRAWINGS

[0089] Figure 1 (SEQ ID NO:1) shows a nucleotide sequence that includes a cDNA sequence encoding the C-terminal part of human PDGF-D (hPDGF-D). The nucleotides which encode for the partial fragment of hPDGF-D are 1 to 198. The deduced partial amino acid sequence of hPDGF-D (66 amino acid residues-SEQ ID NO:2) derived from nucleotides 1 to 198 of Figure 1 is shown in Figure 2;

[0090] Figure 3 (SEQ ID NO:3) shows an extended sequence of a partial human cDNA encoding for the hPDGF-D. The translated cDNA sequence is from nucleotide 1 to 600. The deduced partial amino acid sequence of hPDGF-D (200 residues-SEQ ID NO:4) derived from nucleotides 1 to 600 of Figure 3 is shown in Figure 4;

[0091] Figure 5 shows a still further extended nucleotide sequence of a partial human cDNA. The nucleotides which encode for the 5'-truncated full-length hPDGF-D are 1 to 966 (SEQ ID NO:5). The deduced partial amino acid sequence of hPDGF-D (322 residues-SEQ ID NO:6) derived from nucleotides 1 to 966 of Figure 5 is shown in Figure 6;

[0092] Figure 7 (SEQ ID NO:7) shows the complete nucleotide sequence of cDNA encoding a hPDGF-D(1116 bp) and the deduced amino acid sequence of full-length hPDGF-D encoded thereby which consists of 371 amino acid residues (Figure 8-SEQ ID NO:8);

[0093] Figure 9 shows an amino acid sequence alignment of the hPDGF-D with hPDGF-C (SEQ ID NOs:8 and 32, respectively);

[0094] Figure 10 shows an amino acid sequence alignment of the PDGF/VEGF-homology domain in hPDGF-D with several growth factors belonging to the VEGF/PDGF family (SEQ ID NOs:10-18, respectively);

[0095] Figure 11 shows a phylogenetic tree of several growth factors belonging to the VEGF/PDGF family;

[0096] Figure 12 provides the amino acid sequence alignment of the CUB domain present in hPDGF-D (SEQ ID NO:19) and other CUB domains present in human bone morphogenic protein-1 (hBMP-1, 3 CUB domains CUB1-3) (SEQ ID NOs:20-22, respectively) and in human neuropilin-1 (2 CUB domains) (SEQ ID NOs:23-24, respectively);

[0097] Figure 13 shows the results of the SDS-PAGE analysis of human recombinant PDGF-D under reducing (R) and non-reducing (NR) conditions;

[0098] Figure 14 shows the results of the immunoblot analysis of full-length PDGF-D and PDGF-C under reducing and non-reducing conditions employing affinity-purified rabbit antibodies to full-length PDGF-D;

[0099] Figure 15 provides that results of the relative expression levels of PDGF-D (upper panel) and PDGF-B (lower panel) transcripts in several human tissues as determined by Northern Blot analysis;

[00100] Figure 16 shows PDGF-D expression in the developing kidney of a mouse embryo;

[00101] Figure 17 shows a more detailed view of PDGF-D expression in the developing kidney of a mouse embryo;

[00102] Figure 18 shows a more detailed view of PDGF-D expression in the developing kidney of a mouse embryo;

[00103] Figure 19 shows that conditioned medium(CM)containing plasmin-digested PDGF-D stimulates tyrosine phosphorylation of PDGFR-beta in PAE-1 cells;

[00104] Figure 20 provides a graphical representation of the results of the competitive binding assay between plasmin-digested PDGF-D and PDGF-BB homodimers for the PDGFRs-beta; and

[00105] Figure 21 provides a graphical representation of the results of the competitive binding assay between plasmin-digested PDGF-D and PDGF-AA homodimers for the PDGFRs-alpha.

[00106] Figure 22A shows a schematic representation of the PDGF-D sequence of SEQ ID NO:35.

[00107] Figure 22B shows a schematic representation of the PDGF-D sequence variant of SEQ ID NO:37, which corresponds to Figure 22A but for 6 missing amino acid residues.

[00108] Figure 22C shows a schematic representation of the PDGF-D sequence variant of SEQ ID NO:39, which corresponds to Figure 22A but for 6 missing amino acid residues and the loss of a CUB domain in this sequence variant.

[00109] Figure 23 shows a schematic representation of the PDGF-D sequence, noting the spliced region from exon 5 to exon 7, removal of which yields the PDGF-D sequence variant of SEQ ID NO:39.

[00110] Figure 24 shows SDS-PAGE analysis under reducing conditions of human PDGF-DD formed from the core domain of factor Xa-digested mutant full-length form of PDGF-D.

[00111] Figure 25 shows the *in vivo* angiogenic activity of human PDGF-DD and other PDGF isoforms in the mouse cornea pocket assay. In Fig. 25A-E, arrows point to where PDGF protein-containing beads were implanted.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[00112] Figure 1 shows a nucleotide sequence of human cDNA which encodes a C-terminal portion of a novel growth factor, referred to herein as PDGF-D (formerly VEGF-G). PDGF-D is a new member of the VEGF/PDGF family. The nucleotide sequence of Figure 1 (SEQ ID NO:1) was derived from a human EST sequence (id. AI488780) in the dbEST database at the NCBI in Washington, DC. The nucleotides 1 to 198 of the cDNA of Figure 1 (SEQ ID NO:1) encodes a 66 amino acid polypeptide (Figure 2-SEQ ID NO:2) which shows some sequence similarity to the known members of the VEGF/PDGF family.

[00113] The amino acid sequence of the polypeptide encoded by the nucleotides 1 to 198 of the polynucleotide of Figure 1 (SEQ ID NO:1) is shown in Figure 2 (SEQ ID NO:2).

[00114] To generate more sequence information on human PDGF-D, a human fetal lung □gt10 cDNA library was screened using a 327 bp polymerase chain reaction (PCR)-generated probe, based on the originally identified EST sequence. The probe was generated from DNA from a commercially available human fetal lung cDNA library (Clontech) which was amplified by PCR using two primers derived from the identified EST (AI488780). The primers were:

5'-GTCGTGGAACTGTCAACTGG (forward) (SEQ ID NO:26) and

5'-CTCAGCAACCACTTGTGTTC (reverse) (SEQ ID NO:27).

The amplified 327 bp fragment was cloned into the pCR2.1 vector (Invitrogen). Nucleotide sequencing verified that the insert corresponded to the EST. The screen identified several positive clones. The inserts from two of these clones, clones 5 and 8 were subcloned into pBluescript and subjected to nucleotide sequencing using internal or vector-specific primers. The nucleotide sequences determined were identical in both clones and are shown in Figure 3 (SEQ ID NO:3). The coding region of the 690 bp polynucleotide is nucleotides 1-600 (SEQ ID NO:3) that encodes for a large portion of hPDGF-D with the exception of the 5'-end. This portion of hPDGF-D includes the bioactive fragment of hPDGF-D. The deduced partial amino acid sequence of hPDGF-D (200 residues-SEQ ID NO:4) derived from nucleotides 1 to 600 of Figure 3 (SEQ ID NO:3) is shown in Figure 4 (SEQ ID NO:4).

[00115] Extended nucleotide sequencing of the isolated human PDGF-D cDNA clones from this human fetal lung cDNA library has provided additional sequence. Figure 5 (SEQ ID NO:5) shows a nucleotide sequence of a partial human cDNA (1934 bp) that encodes hPDGF-D. The coding region of the 1934 bp polynucleotide is nucleotides 1 to 966 that encodes for hPDGF-D except for the most 5'-end of the polypeptide. The deduced partial amino acid sequence of hPDGF-D (322 residues-SEQ ID NO:6) derived from nucleotides 1 to 966 of Figure 5 (SEQ ID NO:5) is shown in Figure 6 (SEQ ID NO:6).

[00116] Figure 7 (SEQ ID NO:7) shows a polynucleotide sequence of cDNA encoding a full-length hPDGF-D. The region encoding PDGF-D is 1116 bp. The deduced amino acid sequence of full-length hPDGF-D is 370 amino acid residues (Figure 8-SEQ ID NO:8).

[00117] The sequence for the 5' end of full-length PDGF-D was obtained using Rapid Amplification of cDNA Ends (RACE) PCR, and clones containing cDNA from the human heart (Marathon-ReadyTM cDNA, Clontech, Cat# 7404-1). These cDNA clones have an adaptor sequence attached to the 5' end of each clone, including a site for primer called Adaptor Primer 1 (Clontech):

5' CCATCCTAATACGACTCACTATAGGGC 3'(SEQ ID NO:28).

This primer and a second primer:

'AGTGGGATCCGTTACTGATGGAGAGTTAT 3' (SEQ ID NO:29)

were used to amplify the sequence found at the 5' end of PDGF-D. In the PCR reaction a special polymerase mix was used (Advantage«-GC cDNA PCR Kit, Clontech, Cat# K1907-1). The reaction mix included (in microliters):

Adaptor Primer 1

Gene specific primer	1
Template (Human Heart cDNA)	5
GC-Melt (from the K1907-1 Kit)	5
5xGC cDNA PCR Reaction Buffer	10
50x dNTP mix	1
Sterile H_2O	27
Total	50

[00118] The 5' end of PDGF-D was amplified for 31 cycles, five cycles consisted of 45 seconds denaturation at 94°C and four minutes extension at 72°C, five cycles consisted of 45 seconds denaturation at 94°C and four minutes extension at 70°C, and five cycles consisted of 45 seconds denaturation at 94°C and four minutes extension at 68°C and an initial denaturation step at 94°C for two minutes. From this PCR, an approximately 790 bp long product was obtained. This product was run on a 1% agarose gel, purified (QIAquick gel extraction Kit, Qiagen, Cat # 28706) from the gel, cloned into a vector (TOPO TA Cloning Kit, Invitrogen) and transformed into bacteria (*E. Coli*). Transformed bacteria were plated, and incubated at 37°C overnight. Single colonies were picked and grown in fresh media overnight. Plasmids were prepared (QIAprep Spin Miniprep Kit, Qiagen, Cat# 27106) and sequenced with the plasmid primers, T7 and M13R. The result of this sequencing was that 312 bp of previously unknown PDGF-D sequence was obtained. The rest of the sequence (478 bp) was identical with previously obtained sequence from other PDGF-D cDNA clones.

[00119] Similar to PDGF-C, PDGF-D has a two domain structure with a N-terminal CUB domain (residues 67-167, discussed below) and a C-terminal PDGF/VEGF homology domain (residues 272-362, the core domain). The overall amino acid sequence identity between PDGF-C (SEQ ID NO:32) and PDGF-D (SEQ ID NO:8) is approximately 43 % (Figure 9). The similarities are highest in the distinct protein domains while the N-terminal region, including the

hydrophobic signal sequence, and the hinge region between the two domains display lower identities. A putative signal peptidase cleavage site was identified between residues 22-23. Cleavage results in a protein of 348 residue with a calculated molecular mass (M_r) of 44,000. A single putative site for N-linked glycosylation was identified in the core domain of PDGF-D (residues 276-278).

[00120] Figure 10 shows the amino acid sequence alignment of the PDGF/VEGF-homology domain of PDGF-D (found in the C-terminal region of the polypeptide) with the PDGF/VEGF-homology domains of PDGF/VEGF family members, PDGF-C, PDGF-A, PDGF-B, $VEGF_{165}$, PlGF-2, $VEGF\text{-}B_{167}$, VEGF-C and VEGF-D (SEQ ID NOs:10-18, respectively). Gaps were introduced to optimize the alignment. This alignment was generated using the MEGALIGN alignment tool based on the method of J. Hein, (Methods Enzymol. 1990 183 626-45) The PAM 250 residue weight table is used with a gap penalty of eleven and a gap length penalty of three and a K-tuple value of two in the pairwise alignments. The alignment is then refined manually, and the number of identities are estimated in the regions available for a comparison.

[00121] The alignment shows that the core domain of PDGF-D displays about a 50% identity to the corresponding domain in PDGF-C, and about a 20-23% identity to the core domains in the classical PDGFs and VEGFs. It also shows that PDGF-D has the expected pattern of invariant cysteine residues, involved in inter- and intra-disulfide bonding, a hallmark of members of this family, with

two exceptions. The first exception occurs between cysteine 3 and 4. Normally these two cysteines are spaced by 2 residues. However, similar to PDGF-C, PDGF-D has an unique insertion of three additional amino acids residues, NCG. In total, ten cysteine residues reside in the core domain, including the extreme C-terminal region, suggesting a unique arrangement of the cysteines in the disulfide-bonded PDGF-D dimer. The second is that the invariant fifth cysteine found in the other members of the PDGF/VEGF family is not conserved in PDGF-D. This feature is unique to PDGF-D.

[00122] Based on the amino acid sequence alignments in Figure 10, a phylogenetic tree was constructed and is shown in Figure 11. The data show that the PVHD of PDGF-D forms a subgroup of the PDGFs together with PDGF-C.

CUB DOMAIN

[00123] The N-terminal region of the partial PDGF-D amino acid sequence of Figure 12 (residues 54-171 of SEQ ID NO:8) has a second distinct protein domain which is referred to as a CUB domain (Bork and Beckmann, J. Mol. Biol., 1993 231, 539-545). This domain of about 115 amino acids was originally identified in complement factors C1r/C1s, but has recently been identified in several other extracellular proteins including signaling molecules such as bone morphogenic protein 1 (BMP-1) (Wozney *et al.*, Science, 1988 242, 1528-1534) as well as in several receptor molecules such as neuropilin-1 (NP-1) (Soker *et al.*,

Cell, 1998 92 735-745). The functional roles of CUB domains are not clear but they may participate in protein-protein interactions or in interactions with carbohydrates including heparin sulfate proteoglycans. These interactions may play a role in the proteolytic activation of PDGF-D.

[00124] As shown in Figure 12, the amino acid sequences from several CUB-containing proteins were aligned. The results show that the single CUB domain in human PDGF-D (SEQ ID NO:19) displays a significant identify with the most closely related CUB domains. Sequences from human BMP-1, with 3 CUB domains (CUBs1-3)(SEQ ID NOs:20-22, respectively) and human neuropilin-1 with 2 CUB domains (CUBs1-2)(SEQ ID NOs:23-24, respectively) are shown. This alignment was generated as described above.

Example 1: Expression of human PDGF-D in baculovirus infected Sf9 cells

[00125] The portion of the cDNA encoding amino acid residues 24-370 of SEQ ID NO:8 was amplified by PCR using Taq DNA polymerase (Biolabs). The forward primer used was 5′GATATCTAGAAGCAACCCCGCAGAGC 3′ (SEQ ID NO:33). This primer includes a XbaI site (underlined) for in frame cloning. The reverse primer used was 5′ GCTCGAATTCTAAATGGTGATGGTGATGATG TCGAGGTGGTCTTGA 3′ (SEQ ID NO:34). This primer includes an EcoRI site (underlined) and sequences coding for a C-terminal 6X His tag preceded by an enterokinase site. The PCR product was digested with XbaI and EcoRI and

subsequently cloned into the baculovirus expression vector, pAcGP67A. Verification of the correct sequence of the cloned PCR product was done by nucleotide sequencing. The expression vectors were then co-transfected with BaculoGold linearized baculovirus DNA into Sf9 insect cells according to the manufactures protocol (Pharmingen). Recombined baculovirus were amplified several times before beginning large scale protein production and protein purification according to the manual (Pharmingen).

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[00126] Sf9 cells, adapted to serum free medium, were infected with recombinant baculovirus at a multiplicity of infection of about seven. Media containing the recombinant proteins were harvested four days after infection and were incubated with Ni-NTA-Agarose beads(Qiagen). The beads were collected in a column and after extensive washing with 50 mM sodium phosphate buffer pH 8, containing 300 mM NaCl (the washing buffer), the bound proteins were eluted with increasing concentrations of imidazole (from 100 mM to 500 mM) in the washing buffer. The eluted proteins were analyzed by SDS-PAGE using 12.5% polyacrylamide gels under reducing and non-reducing conditions.

[00127] Figure 13 shows the results of the SDS-PAGE analysis of human recombinant PDGF-D under reducing (R) and non-reducing (NR) conditions. PDGF-D was visualized by staining with Coomassie Brilliant Blue. Figure 13 also shows that the recombinant PDGF-D migrates as a 90 kDa species under

non-reducing conditions and as a 55 kDa species under reducing conditions. This indicates that the protein was expressed as a disulfide-linked homodimer.

Example 2: Generation of antibodies to human PDGF-D

[00128] Rabbit antisera against full-length PDGF-DD and against a synthetic peptide derived from the PDGF-D sequence (residues 254-272, amino acid sequence CRKSKVDLDRLNDDAKRYSC (SEQ ID NO:35)) were generated. These peptides were each conjugated to the carrier protein keyhole limpet hemocyanin (KLH, Calbiochem) using N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Pharmacia Inc.) according to the instructions of the supplier. 200-300 micrograms of the conjugates in phosphate buffered saline (PBS) were separately emulsified in Freunds Complete Adjuvant and injected subcutaneously at multiple sites in rabbits. The rabbits were boostered subcutaneously at biweekly intervals with the same amount of the conjugates emulsified in Freunds Incomplete Adjuvant. Blood was drawn and collected from the rabbits. The sera were prepared using standard procedures known to those skilled in the art. The antibodies to full-length PDGF-DD were affinity-purified on a column of purified PDGF-DD coupled to CNBr-activated Sepharose 4B (Pharmacia).

[00129] As seen in Figure 14, the antibodies did not cross-react with PDGF-C in the immunoblot analysis. For immunoblotting analyses, the proteins were electrotransferred onto Hybond filters for 45 minutes.

Example 3: Expression of PDGF-D transcripts

[00130] To investigate the tissue expression of PDGF-D in several human tissues, a Northern blot was done using a commercial Multiple Tissue Northern blot (MTN, Clontech). The blots were hybridized at according to the instructions from the supplier using ExpressHyb solution at 68°C for one hour (high stringency conditions), and probed sequentially with a ^{32}P-labeled 327 bp PCR-generated probe from the human fetal lung cDNA library (see description above) and full-length PDGF-B cDNA. The blots were subsequently washed at 50°C in 2X SSC with 0.05% SDS for 30 minutes and at 50°C in 0.1X SSC with 0.1% SDS for an additional 40 minutes. The blots were then put on film and exposed at -70°C. As shown in Figure 15, upper panel, the highest expression of a major 4.4 kilobase (kb) transcript occurred in heart, pancreas and ovary while lower expression levels were noted in several other tissues including placenta, liver, kidney, prostate, testis, small intestine, spleen and colon. No expression was detected in brain, lung, skeletal muscle. In comparison, the 3.5 kb PDGF-B transcript was abundantly expressed in heart and placenta, whereas lower levels were observed in all other tissues (Figure 15, lower panel). Prominent co-expression of PDGF-D and PDGF-B occurred in heart, pancreas and ovary.

Example 4: Immunohistochemistry localization of VEGF-D in mouse embryos

[00131] The spatial and temporal patterns of expression of the PDGF-D protein in mouse embryos were determined by immunohistochemistry using

standard procedures and employing affinity-purified rabbit antibodies to full-length PDGF-DD generated in Example 2 on tissue sections of embryos during midgestation (embryonic day (E) 14.5). The embryos were fixed in 4% paraformaldehyde overnight at 4°C and processed for cryosectioning. 14□m cryosections were used for the stainings. Paraffin-embedded sections which were prepared by routine procedures were also used. After sectioning, the slides were air dried for one to three hours followed by a ten minute post fixation with 4% paraformaldehyde. After washing 3 x 5 minutes with phosphate buffered saline (PBS) containing 0.3% Triton X-100 (PBS-T), the slides were incubated in 0.3% H_2O_2 in PBS-T for 30 minutes to quench the endogenous peroxidase activity. This was followed by washing 2 x 5 minutes with PBS-T and 2 x 5 minutes in PBS. Blocking of non-specific binding was done using 3% bovine serum albumin (BSA) in PBS for 30 minutes. The slides were incubated with the affinity purified antibody to human PDGF-DD (3-9 mg of Ig/ml) overnight at 4°C. After washing, the slides were incubated with the secondary Ig (goat anti-rabbit HRP, Vector Laboratories) at a dilution of 1:200 for one hour. After washing, the slides were incubated with the AB complex (Vector Laboratories) for one hour and washed with Tris pH 7.4. Either 3,3'-diaminobenzidine tetrahydrochloride (DAB from SIGMA) or 3-amino-9-ethyl carbazole (AEC from Vector Laboratories) were used for color development. The reaction was quenched by washing in Tris-HCl buffer. In control experiments the antibodies were preincubated with a 30x molar excess of full-length PDGF-DD. This blocked the staining, while a similar

preincubation with full-length PDGF-CC did not affect the staining of the tissue sections. The photomicrographs were taken using a Zeiss microscope equipped with differential interference contrast optics.

[00132] Intense staining for PDGF-D was noted in the developing heart, lung, kidney and some muscle derivatives. Figures 16-18 show the staining of the embryonic kidney. Intense staining of the highly vascularized fibrous capsule (fc) surrounding the kidney, the adjacent adrenal gland (ag), and in the most peripheral aspect of the metanephric mesenchyme (mm) of the cortex was observed (Figures 16 and 17). Staining was also observed in cells located in the basal aspect of the branching ureter (Figure 18), while the developing nephron, including the ureter buds, glomeruli (gl) and Henle's loops, were negative. Previous analysis have shown that PDGFR-beta is expressed by the metanephric mesenchyme and the developing vascular smooth muscle cells and mesangial cells of the developing renal cortex. In contrast, renal expression of PDGF-B is restricted to endothelial cells (Lindahl, P. *et al.*, Development, 1998 125 3313-3322). The non-overlapping patterns of expression of the two PDGFR-beta ligands suggests that PDGF-B and PDGF-D provide distinct signals to PDGFR-beta expressing perivascular cells. This differential localization indicates that PDGF-D might have a paracrine role in the proliferation and/or commitment of PDGFR-beta expressing perivascular progenitor cells of the undifferentiated metanephric mesenchyme. In line with the phenotype of PDGF-B deficient mice, PDGF-B may then provide proliferative signals and spatial clues of the

branching vascular tree of the kidney, thus allowing proliferation and co-recruitment of the PDGFR-beta expressing perivascular cells to form the mesangium of the glomeruli, and the smooth muscle cells of the efferent and afferent arterioles.

[00133] The expression of PDGF-D partially overlaps with the expression of PDGF-C in the cortical area of the developing kidney. The different receptor specificities of PDGF-C and PDGF-D and their apparent inability to form heterodimers indicate that the two novel PDGFs may provide distinct signals for migration and proliferation for at least two different cell populations in the undifferentiated metanephric mesenchyme; either interstitial cell progenitors expressing PDGF alpha-receptor, or the PDGFR-beta expressing perivascular progenitor cells.

[00134] The phenotypic differences in the kidneys of mice lacking PDGFR-alpha and PDGF-A argue for a unique role of PDGF-C in the formation of the renal mesenchyme. Interestingly, a comparison of the PDGFR-beta and PDGF-B deficient mice have not revealed a similar phenotypic discrepancy arguing for, at least partially, redundant roles of PDGF-D and PDGF-B during early stages of kidney development.

Example 5: Receptor binding properties of PDGF-D with the VEGF receptors

[00135] To assess the interactions between PDGF-D and the VEGF receptors, truncated PDGF-D was tested for its capacity to bind to soluble Ig-fusion proteins containing the extracellular domains of human VEGFR-1, VEGFR-2 and VEGFR-3 (Olofsson *et al.*, Proc. Natl. Acad. Sci. USA, 1998 95 11709-11714). An expression vector encoding the PDGF/VEGF homology domain of PDGF-D was generated in the vector pSecTag (Invitrogen). The primers 5'-CCCAAGCTTGAAGATCTTGAGAATAT 3' (forward)(SEQ ID NO:30) and 5'-TGCTCTAGATCGAGGTGGTCTT 3' (reverse) (SEQ ID NO:31) were used to amplify a 429 bp fragment (nucleotides 556 to 966 in Figure 5)(SEQ ID NO:5) encoding amino acid residues 186 to 322 of Figure 6 (SEQ ID NO:6). The fragment was subsequently cloned into a HindIII and XbaI digested expression vector. COS cells were transfected with the expression vector encoding truncated PDGF-D or a control vector using calcium phosphate precipitation. The expressed polypeptide included a C-terminal c-myc tag and a 6X His tag (both derived from the pSecTag vector).

[00136] The Ig-fusion proteins, designated VEGFR-1-Ig, VEGFR-2-Ig and VEGFR-3-Ig, were transiently expressed in human 293 EBNA cells. All Ig-fusion proteins were human VEGFRs. Cells were incubated for 24 hours after transfection, washed with Dulbecco's Modified Eagle Medium (DMEM) containing 0.2% bovine serum albumin (BSA) and starved for 24 hours. The

fusion proteins were then precipitated from the clarified conditioned medium using protein A-Sepharose beads (Pharmacia). The beads were combined with 100 microliters of 10X binding buffer (5% BSA, 0.2% Tween 20 and 10 □g/ml heparin) and 900 microliter of conditioned medium prepared from the COS cells transfected with the expression vector for truncated PDGF-D or the control vector. The cells were then metabolically labeled with ^{35}S-cysteine and methionine (Promix, Amersham) for 4 to 6 hours. After 2.5 hours, at room temperature, the Sepharose beads were washed three times with binding buffer at 4°C, once with phosphate buffered saline (PBS) and boiled in SDS-PAGE buffer. Labeled proteins that were bound to the Ig-fusion proteins were analyzed by SDS-PAGE under reducing conditions. Radiolabeled proteins were detected using a phosphorimager analyzer and/or on film. In all these analyses, radiolabeled PDGF-D failed to show any interaction with any of the VEGF receptors. These results indicate that secreted truncated PDGF-D does not bind to VEGF receptors R1, R2 and R3.

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Example 6: PDGFR-beta Phosphorylation

[00137] To test if PDGF-D causes increased phosphorylation of the PDGFR-beta, full-length and plasmin-digested PDGF-D were tested for their capacity to bind to the PDGFR-beta and stimulate increased phosphorylation.

[00138] A plasmin-digested preparation of PDGF-DD was generated and analyzed since it is known that plasmin-digestion of full-length PDGF-CC

releases the core domain and thus allow the ligand to interact with the receptor. Full length PDGF-DD was digested with plasmin in 20 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.01% Tween 20 for 1.5 to 4.5 hours at 37°C using two to three units of plasmin (Sigma) per ml.

[00139] Analysis of the plasmin-digested preparation of PDGF-DD by SDS-PAGE under reducing conditions showed two prominent bands of 28 kDa and 15 kDa. The 15 kDa band was identified as the core domain due to its immunoreactivity in immunoblotting with a peptide antiserum raised against a sequence of PDGF-D just N-terminal of the first cysteine residue in the core domain.

[00140] Serum-starved porcine aortic endothelial-1 (PAE-1) cells stably expressing the human PDGFR-beta (Eriksson et al., EMBO J, 1992, 11, 543-550) were incubated on ice for 90 minutes with a solution of conditioned media mixed with an equal volume of PBS supplemented with 1 mg/ml BSA and 10ng/ml of PDGF-BB, 300ng/ml or 1200 ng/ml of full length human PDGF-DD homodimers or 300ng/ml or 1200 ng/ml of digested PDGF-DD. The full length and digested PDGF-DD homodimers were produced as described above. Sixty minutes after the addition of the polypeptides, the cells were lysed in lysis buffer (20 mM tris-HCl, pH 7.5, 0.5% Triton X-100, 0.5% deoxycholic acid, 10 mM EDTA, 1 mM orthovanadate, 1 mM PMSF 1% Trasylol). The PDGFR-beta were immunoprecipitated from cleared lysates with rabbit antisera against the human

PDGFR-beta (Eriksson *et al.*, EMBO J, 1992 11 543-550). The precipitated receptors were applied to a SDS-PAGE gel. After SDS gel electrophoresis, the precipitated receptors were transferred to nitrocellulose filters, and the filters were probed with anti-phosphotyrosine antibody PY-20, (Transduction Laboratories). The filters were then incubated with horseradish peroxidase-conjugated anti-mouse antibodies. Bound antibodies were detected using enhanced chemiluminescence (ECL, Amersham Inc). The filters were then stripped and reprobed with the PDGFR-beta rabbit antisera, and the amount of receptors was determined by incubation with horseradish peroxidase-conjugated anti-rabbit antibodies. Bound antibodies were detected using enhanced chemiluminescence (ECL, Amersham Inc). The probing of the filters with PDGFR-beta antibodies confirmed that equal amounts of the receptor were present in all lanes. Human recombinant PDGF-BB (100ng/ml) and untreated cells were included in the experiment as a control. Figure 19 shows plasmin-digested PDGF-DD efficiently induced PDGFR-beta tyrosine phosphorylation. Full-length PDGF-DD failed to induce PDGFR-beta tyrosine phosphorylation. PDGF-BB was included in the experiment as a positive control. This indicates that plasmin-digested PDGF-D is a PDGFR-beta ligand/agonist.

Example 7: Competitive Binding Assay

[00141] Next, full length and plasmin-digested PDGF-D were tested for their capacity to bind to human PDGF alpha- and beta-receptors by analyzing their

abilities to compete with PDGF-BB for binding to the PDGF receptors. The binding experiments were performed on porcine aortic endothelial-1 (PAE-1) cells stably expressing the human PDGF alpha- and beta-receptors, respectively (Eriksson et al., EMBO J, 1992, 11, 543-550). Binding experiments were performed essentially as in Heldin et al. (EMBO J, 1988, 7 1387-1393). Different concentrations of human full-length and plasmin-digested PDGF-DD, or human PDGF-BB were mixed with 5 ng/ml of ^{125}I-PDGF-BB in binding buffer (PBS containing 1 mg/ml of bovine serum albumin). Aliquots were incubated with the receptor expressing PAE-1 cells plated in 24-well culture dishes on ice for 90 minutes. After three washes with binding buffer, cell-bound ^{125}I-PDGF-BB or ^{125}I-PDGF-AA was extracted by lysis of cells in 20 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Triton X-100. The amount of cell bound radioactivity was determined in a gamma-counter. An increasing excess of the unlabeled protein added to the incubations competed efficiently with cell association of the radiolabeled tracer.

[00142] Figure 20 provides a graphical representation of results which show that conditioned medium containing plasmin-digested PDGF-DD competes for binding with PDGF-BB homodimers for the PDGFRs-beta, while the full length protein did not. Compared to PDGF-BB, plasmin-activated PDGF-DD appeared 10-12 fold less efficient as a competitor; probably a result of suboptimal activation of the recombinant protein *in vitro* by the protease. Control experiments showed that plasmin present in the digested PDGF-DD fraction did

not affect the binding of ^{125}I-labelled PDGF-BB to the PDGFR-β-expressing cells. Both the full length and plasmin-digested proteins failed to compete for binding to the PDGFR-alpha (Figure 21).

[00143] These studies indicate that PDGF-DD is a PDGFR-beta-specific agonist and that proteolytic processing releases the core domains of PDGF-DD from the N-terminal CUB domains which is necessary for unmasking the receptor-binding epitopes of the core domain similar to the situation for PDGF-CC.

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Example 8: Determination of Alternative Splicing of Murine PDGF-D

[00144] Primers were designed for the amplification of the whole coding area of murine PDGF-D by PCR from mouse heart cDNA (Clontech). These primers were: 5'-CAAATGCAACGGCTCGTTT-3' (SEQ ID NO:41) and 5'-GATATTTGCTTCTTCTTGCCATGG-3' (SEQ ID NO:42). PCR reaction conditions were as follows: PCR Cycles: 94°C for 2 minutes, followed by 30 cycles: 94°C for 45 seconds, 62°C for 45 seconds, 72°C for 90 seconds, and 72°C for 7 minutes.

[00145] The expected product from this reaction was a 1.2 kb cDNA fragment. However, the product was two bands, one approximately 1.2 kb and the other only 1.0 kb. These two products were checked in a 1% agarose gel, purified from the gel (QIAquick Gel Extraction Kit, Qiagen, Cat # 28706), cloned

into a vector (TOPO TA Cloning Kit, Invitrogen), and transformed into *E. Coli* bacteria.

[00146] Transformed bacteria were plated and incubated at 37°C overnight. The next morning some single colonies were picked and grown in fresh medium overnight. Plasmids were prepared (QIAprep Spin Miniprep Kit, Qiagen, Cat # 27106) and sequenced with plasmid primers T7 and M13R, and also with mPDGF-D specific primers. The results revealed three different types of murine PDGF-D cDNAs, one being completely identical with the earlier mouse clones, depicted in SEQ ID NO: 35.

[00147] The second clone was almost identical to the earlier mouse sequence, however, it lacked six amino acid residues (aa 42-47) from the region between the signal sequence and the CUB domain. The second clone is depicted in SEQ ID NO:37. The third clone was comprised of part of the earlier mouse sequence, lacking amino acids 42-47 as in the second clone, and also lacking the PDGF-homology domain. The third clone is depicted in SEQ ID NO:39. The similarities and differences between regions of the three clones are depicted in Figure 22.

[00148] The surprising results show that at least two alternatively spliced versions of the PDGF-D gene are transcribed into polyadenylated RNA. The variant transcript structures suggest an alternative splice acceptor site is used

in exon two, producing a variant protein lacking six amino acid residues (ESNHLT).

[00149] In addition to lacking the above noted six amino acid residues, the third clone also lacks the PDGF-homology domain. This is because of the skipping of exon six and the resulting frameshift. This ends the open reading frame into a stop codon after four additional amino acid residues (GIEV). As shown in detail in Figure 23, this splice variant only contains the amino terminal CUB domain and could potentially provide an inhibitor of PDGF-D functions. The potential inhibition function is because the activation of full-length PDGF-D binding to the PDGFR-D requires proteolytic removal of the CUB domain.

Example 9: Generation of Recombinant human PDGF-DD core domain

[00150] The process as described (Bergsten et al., 2001, Nat. Cell Biol. 3: 512-516) was followed to generate recombinant human PDGF-DD core domain. Human PDGF-DD was expressed as a mutant full-length form containing a factor Xa protease cleavage site that allowed the generation of the active C-terminal fragment of the protein (PDGF-homology) upon cleavage with factor Xa. The recombinant protein has an extreme C-terminal His_6-tag to allow its purification on a nickel-containing resin. Following purification, the protein solution was dialyzed against 0.1M acetic acid and lyophilized. SDS-PAGE analysis under reducing conditions on the purified protein revealed that it

migrated as a homogenous 21 kDa species (Fig. 24). The purified protein was lyophilized for storage.

Example 10: Comparison of Angiogenic Activities of the human PDGF-DD Core Domain with Other PDGF Isoforms.

[00151] The mouse corneal micropocket assay was performed according to procedures described in Cao et al., Proc Natl Acad Sci USA 1998 95 14389-94; Cao et al., Nature 1999 398 381. Specifically, lyophilized proteins were dissolved in phosphate buffer solutions (PBS) and used to make protein bound polymer beads, as described.

[00152] The beads were then implanted in mouse cornea. Male 5-6 week-old C57BI6/J mice were acclimated and caged in groups of six or less. Animals were anaesthetized by injection of a mixture of dormicum and hypnorm (1:1) before all procedures. Corneal micropockets were created with a modified von Graefe cataract knife in both eyes of each male 5-6-week-old C57BI6/J mouse. A micropellet (0.35 x 0.35 mm) of sucrose aluminum sulfate (Bukh Meditec, Copenhagen, Denmark) coated with slow-release hydron polymer type NCC (IFN Sciences, New Brunswick, NJ) containing various amounts of homodimers of truncated PDGF-DD was surgically implanted into each cornal pocket.

[00153] For comparison purposes corresponding amounts of PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC were similarly implanted into corneal pockets of test mice. In each case, the pellet was positioned 0.6-0.8 mm from the corneal

limbus. After implantation, erythromycin/ophthalmic ointment was applied to each eye.

[00154] On day 5 after growth factor implantation, animals were sacrificed with a lethal dose of CO_2, and corneal neovascularization was measured and photographed with a slit-lamp stereomicroscope. In Fig. 25 A-E, arrows point to the implanted pellets. Vessel length and clock hours of circumferential neovascularization were measured. Quantitation of corneal neovascularization is presented as maximal vessel length (Fig. 25F), clock hours of circumferential neovascnlarization (Fig. 25G), and area of neovascularization (Fig. 25H). Graphs represent mean values (Å SEM) of 11-16 eyes (6-8 mice) in each group.

[00155] The corneal angiogenesis model is one of the most rigorous mammalian angiogenesis models that requires a putative compound to be sufficiently potent in order to induce neovascularization in the corneal avascular tissue. Potent angiogenic factors including FGF-2 and VEGF have profound effects in this system.

[00156] The results are shown in Figure 25. The assays were done using PDGF-AA(Fig. 25A), PDGF-AB(Fig. 25B), PDGF-BB(Fig. 25C), PDGF-CC(Fig. 25D), and PDGF-DD(Fig. 25C). Figure 25F-H show the quantitative analysis of vessel length, clock hours, and vessel areas (means ± SD, n=4-6).

[00157] The overall angiogenic response induced by PDGF-DD was similar to that induced by other PDGF isoforms. The results again clearly demonstrate

that the truncated PDGF-D homodimer exhibits marked angiogenic activity *in vivo*. In light of the foregoing test results, which demonstrate the *in vivo* angiogenesis inducing activity of PDGF-DD, treatments with PDGF-DD alone, or in combination with other angiogenic factors such as VEGF family members and FGFs, provide an attractive approach for therapeutic angiogenesis of ischemic heart, brain and limb disorders.

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BIOASSAYS TO DETERMINE THE FUNCTION OF PDGF-D

[00158] Assays are conducted to evaluate whether PDGF-D has similar activities to PDGF-A, PDGF-B, VEGF, VEGF-B, VEGF-C and/or VEGF-D in relation to growth and/or motility of connective tissue cells, fibroblasts, myofibroblasts and glial cells; to endothelial cell function; to angiogenesis; and to wound healing. Further assays may also be performed, depending on the results of receptor binding distribution studies.

I. Mitogenicity of PDGF-D for Endothelial Cells

[00159] To test the mitogenic capacity of PDGF-D for endothelial cells, the PDGF-D polypeptide is introduced into cell culture medium containing 5% serum and applied to bovine aortic endothelial cells (BAEs) propagated in medium containing 10% serum. The BAEs are previously seeded in 24-well dishes at a density of 10,000 cells per well the day before addition of the PDGF-D. Three

days after addition of this polypeptide the cells were dissociated with trypsin and counted. Purified VEGF is included in the experiment as positive control.

II. Mitogenicity of PDGF-D for Fibroblasts

[00160] To test the mitogenic capacity of PDGF-D for fibroblasts, different concentrations of truncated homodimers of PDGF-DD or PDGF-AA (as control) are added to serum starved human foreskin fibroblasts in the presence of 0.2 □mCi [3H]thymidine. The fibroblasts are then incubated for 24 hours with 1 ml of serum-free medium supplemented with 1 mg/ml BSA. After trichloroacetic acid (TCA) precipitation, the incorporation of [3H]thymidine into DNA is determined using a beta-counter. The assay is performed essentially as described in Mori *et al.*, J. Biol. Chem., 1991 266 21158-21164.

III. Assays of Endothelial Cell Function

a) *Endothelial cell proliferation*

[00161] Endothelial cell growth assays are performed by methods well known in the art, *e.g.* those of Ferrara & Henzel, Nature, 1989 380 439-443, Gospodarowicz *et al.*, Proc. Natl. Acad. Sci. USA, 1989 86 7311-7315, and/or Claffey *et al.*, Biochem. Biophys. Acta, 1995 1246 1-9.

b) *Cell adhesion assay*

[00162] The effect of PDGF-D on adhesion of polymorphonuclear granulocytes to endothelial cells is tested.

c) *Chemotaxis*

[00163] The standard Boyden chamber chemotaxis assay is used to test the effect of PDGF-D on chemotaxis.

d) *Plasminogen activator assay*

[00164] Endothelial cells are tested for the effect of PDGF-D on plasminogen activator and plasminogen activator inhibitor production, using the method of Pepper *et al.*, Biochem. Biophys. Res. Commun., 1991 181 902-906.

e) *Endothelial cell Migration assay*

[00165] The ability of PDGF-D to stimulate endothelial cells to migrate and form tubes is assayed as described in Montesano *et al.*, Proc. Natl. Acad. Sci. USA, 1986 83 7297-7301. Alternatively, the three-dimensional collagen gel assay described in Joukov *et al.*, EMBO J., 1996 15 290-298 or a gelatinized membrane in a modified Boyden chamber (Glaser *et al.*, Nature, 1980 288 483-484) may be used.

IV. ANGIOGENESIS ASSAY

[00166] The ability of PDGF-D to induce an angiogenic response in chick chorioallantoic membrane is tested as described in Leung *et al.*, Science, 1989

246 1306-1309. Alternatively the rat cornea assay of Rastinejad *et al.*, Cell, 1989 56 345-355 may be used; this is an accepted method for assay of *in vivo* angiogenesis, and the results are readily transferrable to other *in vivo* systems.

V. WOUND HEALING

[00167] The ability of PDGF-D to stimulate wound healing is tested in the most clinically relevant model available, as described in Schilling *et al.*, Surgery, 1959 46 702-710 and utilized by Hunt *et al.*, Surgery, 1967 114 302-307.

VI. THE HEMOPOIETIC SYSTEM

[00168] A variety of *in vitro* and *in vivo* assays using specific cell populations of the hemopoietic system are known in the art, and are outlined below. In particular a variety of *in vitro* murine stem cell assays using fluorescence-activated cell sorter to purified cells are particularly convenient:

a) *Repopulating Stem Cells*

[00169] These are cells capable of repopulating the bone marrow of lethally irradiated mice, and have the Lin^-, Rh^{hl}, Ly-$6A/E^+$, c-kit^+ phenotype. PDGF-D is tested on these cells either alone, or by co-incubation with other factors, followed by measurement of cellular proliferation by ^3H-thymidine incorporation.

b) *Late Stage Stem Cells*

[00170] These are cells that have comparatively little bone marrow repopulating ability, but can generate D13 CFU-S. These cells have the Lin^-, Rh^{hl}, Ly-6A/E^+, c-kit^+ phenotype. PDGF-D is incubated with these cells for a period of time, injected into lethally irradiated recipients, and the number of D13 spleen colonies enumerated.

c) *Progenitor-Enriched Cells*

[00171] These are cells that respond *in vitro* to single growth factors and have the Lin^-, Rh^{hl}, Ly-6A/E^+, c-kit^+ phenotype. This assay will show if PDGF-D can act directly on haemopoietic progenitor cells. PDGF-D is incubated with these cells in agar cultures, and the number of colonies present after 7-14 days is counted.

VII. ATHEROSCLEROSIS

[00172] Smooth muscle cells play a crucial role in the development or initiation of atherosclerosis, requiring a change of their phenotype from a contractile to a synthetic state. Macrophages, endothelial cells, T lymphocytes and platelets all play a role in the development of atherosclerotic plaques by influencing the growth and phenotypic modulations of smooth muscle cell. An *in vitro* assay using a modified Rose chamber in which different cell types are seeded on to opposite cover slips measures the proliferative rate and phenotypic

modulations of smooth muscle cells in a multicellular environment, and is used to assess the effect of PDGF-D on smooth muscle cells.

VIII. METASTASIS

[00173] The ability of PDGF-D to inhibit metastasis is assayed using the Lewis lung carcinoma model, for example using the method of Cao *et al.*, J. Exp. Med., 1995 182 2069-2077.

IX. Migration of Smooth Muscle Cells

[00174] The effects of the PDGF-D on the migration of smooth muscle cells and other cells types can be assayed using the method of Koyama *et al.*, J. Biol. Chem., 1992 267 22806-22812.

X. CHEMOTAXIS

[00175] The effects of the PDGF-D on chemotaxis of fibroblast, monocytes, granulocytes and other cells can be assayed using the method of Siegbahn *et al.*, J. Clin. Invest., 1990 85 916-920.

XI. PDGF-D IN OTHER CELL TYPES

[00176] The effects of PDGF-D on proliferation, differentiation and function of other cell types, such as liver cells, cardiac muscle and other cells, endocrine

cells and osteoblasts can readily be assayed by methods known in the art, such as ^{3}H-thymidine uptake by *in vitro* cultures.

XII. CONSTRUCTION OF PDGF-D VARIANTS AND ANALOGUES

[00177] PDGF-D is a member of the PDGF family of growth factors which exhibits a high degree of homology to the other members of the PDGF family. PDGF-D contains seven conserved cysteine residues which are characteristic of this family of growth factors. These conserved cysteine residues form intra-chain disulfide bonds which produce the cysteine knot structure, and inter-chain disulfide bonds that form the protein dimers which are characteristic of members of the PDGF family of growth factors. PDGF-D interacts with a protein tyrosine kinase growth factor receptor.

[00178] In contrast to proteins where little or nothing is known about the protein structure and active sites needed for receptor binding and consequent activity, the design of active mutants of PDGF-D is greatly facilitated by the fact that a great deal is known about the active sites and important amino acids of the members of the PDGF family of growth factors.

[00179] Published articles elucidating the structure/activity relationships of members of the PDGF family of growth factors include for PDGF: Oestman *et al.*, J. Biol. Chem., 1991 266 10073-10077; Andersson *et al.*, J. Biol. Chem., 1992 267 11260-1266; Oefner *et al.*, EMBO J., 1992 11 3921-3926; Flemming *et al.*,

Molecular and Cell Biol., 1993 13 4066-4076 and Andersson *et al.*, Growth Factors, 1995 12 159-164; and for VEGF: Kim *et al.*, Growth Factors, 1992 7 53-64; Pötgens *et al.*, J. Biol. Chem., 1994 269 32879-32885 and Claffey *et al.*, Biochem. Biophys. Acta, 1995 1246 1-9. From these publications it is apparent that because of the eight conserved cysteine residues, the members of the PDGF family of growth factors exhibit a characteristic knotted folding structure and dimerization, which result in formation of three exposed loop regions at each end of the dimerized molecule, at which the active receptor binding sites can be expected to be located.

[00180] Based on this information, a person skilled in the biotechnology arts can design PDGF-D mutants with a very high probability of retaining PDGF-D activity by conserving the eight cysteine residues responsible for the knotted folding arrangement and for dimerization, and also by conserving, or making only conservative amino acid substitutions in the likely receptor sequences in the loop 1, loop 2 and loop 3 region of the protein structure.

[00181] The formation of desired mutations at specifically targeted sites in a protein structure is considered to be a standard technique in the arsenal of the protein chemist (Kunkel *et al.*, Methods in Enzymol., 1987 154 367-382). Examples of such site-directed mutagenesis with VEGF can be found in Pötgens *et al.*, J. Biol. Chem., 1994 269 32879-32885 and Claffey *et al.*, Biochem. Biophys. Acta, 1995 1246 1-9. Indeed, site-directed mutagenesis is so common that kits

are commercially available to facilitate such procedures (*e.g.* Promega 1994-1995 Catalog., Pages 142-145).

[00182] The connective tissue cell, fibroblast, myofibroblast and glial cell growth and/or motility activity, the endothelial cell proliferation activity, the angiogenesis activity and/or the wound healing activity of PDGF-D mutants can be readily confirmed by well established screening procedures. For example, a procedure analogous to the endothelial cell mitotic assay described by Claffey *et al.*, (Biochem. Biophys. Acta., 1995 1246 1-9) can be used. Similarly the effects of PDGF-D on proliferation of other cell types, on cellular differentiation and on human metastasis can be tested using methods which are well known in the art.

[00183] The foregoing description and examples have been set forth merely to illustrate the invention and are not intended to be limiting. Since modifications of the disclosed embodiments incorporating the spirit and substance of the invention may occur to persons skilled in the art, the invention should be construed broadly to include all variations falling within the scope of the appended claims and equivalents thereof.